The di- and tripeptide transport system of Lactococcus lactis

Hagting, Anja

Publication date:
1997

Citation for published version (APA):
Hagting, A. (1997). The di- and tripeptide transport system of Lactococcus lactis. s.n.

5

SUMMARY AND CONCLUDING REMARKS

Peptide transport is occurring in most if not all living organisms, and, in addition to satisfying the organisms amino acid requirements, it may fulfil a variety of other functions (Chapter 1). In the lactic acid bacterium *Lactococcus lactis* peptides are used as sources of amino acids but they may also have signalling functions. For growth in milk, *L. lactis* possesses a proteolytic system to breakdown the milk proteins (caseins) to peptides and to translocate the products across the cytoplasmic membrane. The peptides are subsequently degraded by a multitude of peptidases. At the start of this study, two peptide transport activities were known in *L. lactis*: one for the transport of oligopeptides and one for translocation of di- and tripeptides. Further analysis revealed that these two transport activities were distinct in their biochemical properties (this study; Kunji *et al.*, 1993). Oligopeptides of four upto at least twelve amino acid residues were transported in an ATP-dependent manner by an *o*ligo*p*eptide *p*ermease (Opp), while translocation of di- and tripeptides were ascribed to a proton motive force-driven peptide transport system (DtpT). In this thesis, the molecular properties of the proton motive force-driven peptide transport system DtpT are described.

The *dtpT* gene encoding the peptide transport protein was cloned by complementation of a dipeptide transport deficient and proline auxotrophic *E. coli* strain (Chapter 2). The functional expression of the *dtpT* gene in *E. coli* was demonstrated by uptake studies of radiolabeled peptides that were synthesized from [^{14}C]-labeled amino acids (Chapters 2 and 4).

The flanking regions of the di- and tripeptide transport gene were used to delete *dtpT* from the chromosome of *L. lactis,* and transport assays showed that DtpT is the only transport protein for hydrophilic di- and tripeptides in *L. lactis*. However, an *opp/dtpT* double mutant could still grow on hydrophobic di- and tripeptides, indicating the presence of a third peptide transport system (DtpP) (Foucaud *et al.*, 1995). Deletion of the *dtpT* gene had no effect on the ability of *L. lactis* to grow in milk, while mutant analysis showed that the Opp system is crucial for obtaining essential and growth stimulating amino acids (Kunji *et al.*, 1995).

The nucleotide sequence of *dtpT* was determined and the encoded protein showed no homology to other bacterial peptide transport systems. All genetically characterized bacterial peptide transporters thus far belong to the ABC transporter family. DtpT, however, shares similarity to eukaryotic pmf-driven peptide transporters, e.g., from kidney and small-intestine of man, rabbit and rat.

A secondary structure model of DtpT was proposed by identification of putative transmembrane spanning segments (TMSs) by hydropathy profiling and by application of the 'positive inside rule' (von Heijne and Gavel, 1988; Chapter 3). The 'positive inside rule' states that short cytoplasmic loops have more basic residues than expected from a random distribution in hydrophilic regions of membrane proteins. All the algorithms suggested that DtpT is composed of 12 membrane spanning helices, but the positions of the TMSs and the location of the amino- and carboxyl termini were by no means conclusive. Therefore, the secondary structure model was tested by studying the activity of carboxyl terminal-truncated versions of DtpT fused to the compartment specific reporter proteins alkaline phosphatase and β-galactosidase. Alkaline phosphatase is enzymatically active when it is translocated to the periplasm, while fusions to a cytoplasmic domain are inactive. In contrast, hybrid proteins containing β-galactosidase exhibit reciprocal behaviour. Data obtained from the analysis of the fusion proteins were substantiated by studying the accessibility of cysteine residues by sulfhydryl reagents. For this purpose, single cysteine residues were engineered in cytoplasmic or extracellular loops of DtpT. Only membrane permeable reagents can react with cysteine residues located in cytoplasmic loops, while membrane impermeable reagents can be used to detect the cysteine residues which are located on the outside of the cytoplasmic membrane. Together, these studies demonstrate that DtpT consists of 12 transmembrane spanning segments with a short amino- and a large carboxyl-terminus, both located at the cytoplasmic site of the membrane.

An alignment of DtpT and other pmf-driven peptide transporters was made based on sequence comparisons and hydropathy profiling

(Chapter 3). From this alignment it seems likely that the structure model of the amino-terminal half of DtpT also holds for the eukaryotic peptide transporters and some other putative peptide transporters. The highest similarity in sequence was found in the part between TMS II and VI, which also contains the 'signature motif' of this transport protein family. It is not known what the function of this motif is, but it may play an important role in the transport process. The carboxyl-terminal half of DtpT differs considerably from that of eukaryotic peptide transport proteins.

To study the transport mechanism of DtpT in further detail, the protein was purified and reconstituted in artificial membranes (liposomes) (Chapter 4). The expression levels of DtpT were increased in *L. lactis* by the use of a low copy number vector and by selecting appropriate growth conditions to obtain more protein. A histidine-tag was engineered at the carboxyl terminus of DtpT to make purification by Ni^{2+}-chelate affinity chromatography possible. In a comparison of a variety of different detergents, *n*-dodecyl-β-D-maltoside was found to be the most suitable detergent to solubilize DtpT from membrane vesicles. Addition of extra salt and lipids improved the solubilization and purification of the protein. Purified DtpT was added to liposomes which were saturated with detergent. Upon removal of the detergent, DtpT was reconstituted unidirectionally and in an active state.

The availability of the sequence of *dtpT*, the deletion mutant(s), and the developed methods to reconstitute the purified protein allow us to address structure-function relationships in the protein in the near future, which could help to solve remaining questions.

What is the role of DtpT in the proteolytic system of *L. lactis?* Mutant analysis has shown that DtpT is not crucial for accumulation of casein-derived amino acids *per se*. It might be that DtpT plays a role in regulating the expression of other components of the proteolytic system. The expression of the extracellular proteinase PrtP and its maturation factor PrtM are repressed by the addition of the dipeptides Leu-Pro and Pro-Leu to the growth medium (Marugg *et al.*, 1995). In mutants lacking di/tripeptide transport, the repression by these peptides was not observed. The activity of the intracellular peptidases PepXP and PepN is in some strains regulated in a comparable manner (Meijer *et al*., 1996). DtpT might, therefore, act as translocator or sensor of small signalling or effector peptides.

Another intriguing observation is that DtpT can transport a large variety of substrates, differing in composition and charge content. From an energetic point of view, it would be important to know the number of protons used in the transport process. If transport always occurs in an electrogenic manner, i.e. when net positive charge is translocated into the cell, the amount of protons transported must depend on the overall charge of the peptide. The transport protein must therefore be capable to accept a variable number of protons or allow the protons to be carried by the substrate. If one assumes the same stoichiometry, i.e., the same number of protons per transported substrate, indifferent of the charge of the peptide, transport must be driven by other forces than the $\Delta\psi$ in case of multiply negatively charged peptides. In this case, transport would be driven by the concentration gradient of the substrate and the pH gradient, and counteracted by the membrane potential.

How does DtpT bind and translocate its substrates? The binding of peptides may involve similar principles as described for the peptide binding proteins of the ABC-type transporters, i.e., binding of the backbone while the side chains are accommodated in pockets. The nature of the pocket might contribute to a preference for certain side chains. Is there a critical role for some specific histidyl residues in H^+ binding and translocation as has been suggested for the renal and intestinal mammalian peptide transporters (Fei *et al.*, 1997)? The secondary structure model proposed in Chapter 3 and the multiple alignment of DtpT with other members of this transporter family can be helpful in designing site-directed mutations in the DtpT protein.

At the same time, the level of expression and ease of purification of DtpT is such that crystallographic studies are well within reach. The requirements for the structural analysis that have been met include; (i) the protein can be obtained in large quantities from its natural source, (ii) a purification scheme has been worked out, and (iii) functional reconstitution in a unidirectional manner has been achieved.

(For references: see summary in Dutch.)